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In polarized epithelial cells, components of the membrane fusion machinery, the t-SNAREs syntaxin 2, 3, 4 and SNAP-23 are differentially localized at the apical and/or basolateral plasma membrane domains. Surprisingly, all of these t-SNAREs redistribute to intracellular locations when MDCK or mammary cells lose their cellular polarity. Apical SNAREs re-localize to the previously characterized vacuolar apical compartment (VAC) while basolateral SNAREs redistribute to a novel organelle that appears to be the basolateral equivalent of the VAC. Both 'intracellular plasma membrane compartments' are associated with the actin cytoskeleton and receive membrane traffic from cognate apical or basolateral pathways, respectively. These findings demonstrate a fundamental shift in plasma membrane traffic towards intracellular compartments while protein sorting is preserved when epithelial cells lose their cell polarity.

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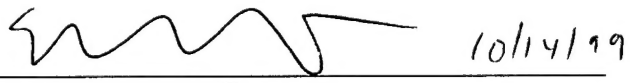

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5. Introduction

In polarized epithelial cells, components of the membrane fusion machinery, the t-SNAREs syntaxin 2, 3, 4 and SNAP-23 are differentially localized at the apical and/or basolateral plasma membrane domains. Surprisingly, all of these t-SNAREs redistribute to intracellular locations when cells lose their cellular polarity. Apical SNAREs re-localize to the previously characterized vacuolar apical compartment (VAC) while basolateral SNAREs redistribute to a novel organelle that appears to be the basolateral equivalent of the VAC. Both 'intracellular plasma membrane compartments' are associated with the actin cytoskeleton and receive membrane traffic from cognate apical or basolateral pathways, respectively. These findings demonstrate a fundamental shift in plasma membrane traffic towards intracellular compartments while protein sorting is preserved when epithelial cells lose their cell polarity.

6. Body

Traffic between membranous compartments is mediated by the SNARE machinery in virtually all membrane traffic pathways investigated so far (14, 15, 27, 33). During vesicle docking, membrane proteins on the vesicle (v-SNAREs) and the target membrane (t-SNAREs) bind to each other to form a complex which ultimately leads to fusion of the lipid bilayers. One aspect of the SNARE hypothesis is that successful membrane fusion requires the binding of matching combinations of v- and t-SNAREs thereby ensuring the necessary specificity of vesicle fusion. Accordingly, each membrane organelle and each class of transport vesicles should be defined by a certain set of t- and v-SNARE isoforms. Many SNAREs have been identified to date and protein sequence analysis has shown that v- and t-SNAREs of the currently known SNARE sub-families are evolutionarily related to each other and belong to a common superfamily (41, 43). It is conceivable that the specificity of vesicle fusion is not directly determined by t-SNARE/v-SNARE interactions *per se* but rather by interactions involving larger complexes including SNAREs and their regulatory proteins such as those of the rab and sec1 protein families (4, 12, 28).

Epithelial cells, including mammary cells, display an additional layer of complexity as they are typically polarized and possess two distinct plasma membrane domains (6, 18, 20, 34, 44). The apical and basolateral plasma membrane domains have different protein and lipid compositions which reflect the different function of these domains. This plasma membrane polarity is established and maintained by protein sorting and specific vesicle trafficking routes in the biosynthetic and endocytic pathways. In agreement with the SNARE hypothesis, the apical and basolateral plasma membrane domains of epithelial cells contain distinct t-SNAREs (42). Two protein families have been identified as t-SNAREs, the syntaxin and SNAP-25 families. In MDCK

cells, syntaxins 3 and 4 are localized at the apical or basolateral plasma membrane, respectively (21). Syntaxin 3 functions in transport from the trans-Golgi network as well as the endosomal recycling pathway, both leading to the apical plasma membrane (22). Syntaxin 2 is localized to both domains of MDCK cells (21), as is SNAP-23 (22), a ubiquitously expressed member of the SNAP-25 family (30). SNAP-23 binds to syntaxins 3 and 4 in vivo (10, 36) and is involved in biosynthetic and endocytic recycling and transcytotic pathways to both plasma membrane domains in MDCK cells (19, 23). The subcellular localization of these SNAREs is generally very similar in other epithelial cell lines and tissues although variations have been reported (5, 8-10, 32, 42).

Temporary or permanent loss of cell polarity is a common phenomenon during the development of epithelial tissues (1, 35) as well as in a number of pathological conditions (1, 7, 20). It is largely unknown how apical and basolateral membrane traffic pathways behave in epithelial cells that have lost or not yet acquired their cellular polarity under any of the above circumstances. This is a fundamental question in cell biology and in the biology of epithelial cancers, such as breast cancer. For example, changes in these pathways may play an important role in the acquisition of the invasive phenotype of tumor cells, e.g. by mistargeting of cell adhesion molecules, or erroneous secretion of proteases that attack basement membrane and extracellular matrix proteins. It is well established that the malignancy of epithelial-derived tumors (carcinomas) correlates directly with the degree of de-differentiation. A hallmark of de-differentiation or anaplasia is the loss of cellular polarity. A better knowledge of the changes in membrane traffic pathways that occur when epithelial cells lose or gain cell polarity will help us understand normal epithelial function as well as pathologic conditions.

In the present grant we have investigated the subcellular localization of plasma membrane t-SNAREs, as part of the machinery accomplishing membrane traffic, in polarized vs. non-polarized cells.

We studied localization of SNAREs in several lines of epithelial cells, where we could manipulate their state of polarity. For mammary cells, we used the 31EG4 cell line, described below. For comparison, we used Madin Darby canine kidney (MDCK) cells, as trafficking and SNARE localization have been very well described in these cells. Basically, we have found almost identical results for both cell types. The description of results below focuses primarily on MDCK cells.

The polarity of the 31EG4 cells was manipulated by several treatments, such as glucocorticoids and TGFbeta. Glucocorticoids, TGF-β, c-Fos and c-Jun are important regulators of both normal mammary development as well as tumorigenesis, and their effects on IM-2 derived cell lines have been examined. Glucocorticoids were initially found to be necessary for the expression of caseins and whey acidic protein in mammary gland explants (reviewed by (33)). Using 31EG4 cells, Zettl et al. (30) defined a new role for glucocorticoids. Cell monolayers grown on permeable

supports in the presence of dexamethasone, a synthetic glucocorticoid, resulted in a 64-fold increase in TER and a two fold increase in the expression of the tight junction protein ZO-1. Dexamethasone, in the presence of prolactin, increased the functional polarity of the cell surface (34). This was the first demonstration of an induction of membrane protein polarity of a epithelial cell. Similar results have since been described for Con8 cells, a rat mammary tumor cell line . Growth of Con8 cells was strongly inhibited by dexamethasone (35). When Con8 cells were grown on permeable supports, dexamethasone caused an increase in the TER and the appearance of a more regular monolayer (36). The exogenous addition of TGF-α to the basolateral, but not the apical membrane domain on Con8 cells grown on permeable supports was able to over-ride the growth inhibition of dexamethasone (12). It appeared that the effect of glucocorticoids on this tumorigenic cell line was to induce differentiation. This represents the second example of glucocorticoids regulating the differentiation and function polarity of a membrane bound protein. Examining the changes that take place in the cellular machinery involved in protein trafficking in response to glucocorticoids will help to evaluate the degree of de-differentiation a given tumor has undergone and to define the requirements of the epithelial cell for polarity.

TGF-β and glucocorticoids have antagonistic effects on 31EG4 cells, dependent on the length of time the cells were previously exposed to glucocorticoids (37). When added to mouse mammary gland epithelial cells, NMuMG, TGF-β induced the transdifferentiation of epithelial cells to mesenchymal cells (38). R. Derynck's lab found that NMuMG cells decreased their expression of epithelial markers E-cadherin, ZO-1, and desmoplakins I and II, and increased the expression of the mesenchymal marker fibronectin. (Dr. Derynck will serve as a consultant to this proposal; see included letter). Over-expression of TGF-β in mammary gland during pregnancy inhibited development of lobuloalveolar structures and suppressed production of milk proteins (39). Therefore the effects of TGF-β on protein trafficking and syntaxin expression should be representative of the physiological changes during mammary involution.

The AP-1 nuclear transcription factors fall into two distinct groups: c-Jun and c-Fos. c-Fos members are only able to form heterodimers with c-Jun members, whereas c-Jun members can form both homodimers with other members or heterodimers with other Fos members (40). Over-expression of c-Fos will drive the formation of heterodimers. Over-expression of c-Jun will drive the formation of homodimers. Studies on at least one promoter element, the plfG composite element of the protein, proliferin, indicate that it is stimulated by the glucocorticoid receptor when Jun/Jun homodimers are bound, but repressed when Fos/Jun heterodimers are bound. There are many examples of genes that are activated by Fos/Jun and repressed by the glucocorticoid receptor. In this way, growth factors that work by stimulating the expression of Fos or Jun can affect the expression of many different gene products. The interaction between the glucocorticoid receptor and AP-1 is again indicative of how important glucocorticoids are, and how the ablation of hormones at the end of lactation signals the large change in gene expression in involved in involution

Overexpression of Fos or Fos and Jun in epithelial clones from IM-2 cells inhibited the ability of the lactogenic hormones to stimulate β-casein (31, 32). In turn, glucocorticoids were found to decrease endogenous AP-1 expression levels. Prolonged expression of Fos caused an irreversible loss of cell polarity and TER (32). The apical marker dipeptidyl peptidase IV (DPP IV) became randomly expressed over the entire cell surface, while the normally basolateral cell adhesion protein, E-cadherin, was first localized apically as well as basolaterally and then later was absent. Cell morphology became fibroblastoid with the eventual expression of the mesenchymal markers, vimentin and fibronectin. To regulate the expression of c-Fos and c-Jun in these studies, they were expressed as fusion proteins with the estrogen receptor (designated c-FosER and c-JunER). Binding of estrogen to the fusion protein caused activation of the c-Fos or c-Jun (31, 41).

The expression of c-JunER in epithelial cells derived from IM-2 cells resulted in a less severe loss of epithelial cell polarity (41). Unlike the case with c-FosER, cell polarity was recovered after removal of estrogen. Activation of c-JunER caused the redistribution of the apical marker DPP IV along with normally apically restricted microvilli to form all over the cell surface, loss of TER, and the down regulation of E-cadherin and ZO-1. Removal of estrogen resulted in the return of monolayer TER, which is a good indication of a well polarized monolayer. Therefore, the effects initiated by the overexpression of c-Jun maybe easier to decipher. The cell is induced to transdifferentiate by Fos/Jun,. Instead the transactivating events initiated by Jun/Jun homodimers predominate, and the cell undergoes a more limited loss of cell polarity. This may represent the first step toward tumorigenesis.

In summary, alterations in mammary epithelial cell polarity in response to glucocorticoids (increased polarity), TGF-β (decreased polarity, but dependent on prior exposure to glucocorticoids), c-jun (reversible loss in cell polarity), and c-fos (irreversible loss of polarity) reflect a spectrum of differentiation and polarization.

We. observed that the previously characterized plasma membrane t-SNAREs in MDCK and mammary cells undergo similar dramatic changes in subcellular localization depending on the degree of cellular polarity. Cells were plated at high density onto polycarbonate filters and the localization of syntaxins 2, 3, 4, and SNAP-23, as well as the tight junction protein ZO-1, were monitored at different times after plating. After 2 hours, the cells are irregularly shaped and start to form cell-cell contacts. At this stage, all plasma membrane t-SNAREs are found predominantly in intracellular vesicles in addition to a variable amount of plasma membrane staining. In approximately 10% of the cells, large intracellular vacuolar structures can be observed. After one day, the monolayer is confluent and uninterrupted circumferential tight junctions are established. A substantial portion of all SNAREs has re-localized to the plasma membrane in a polarized manner. Syntaxins 2 as well as SNAP-23 are found at both the basolateral and apical plasma membrane in addition to some remaining intracellular labeling. Syntaxin 3 is absent from the basolateral domain but localizes to the apical domain in addition to intracellular lysosomes as

established previously (5, 21). Syntaxin 4, in turn, is absent from the apical domain but has partially re-localized to the basolateral domain. During the course of the experiment, until day 7, the cells grow somewhat in height and form a straight apical surface. All of the SNAREs continue to move to their final destination at their specific plasma membrane domains, however, even after 7 days some intracellular staining remains in each case as observed previously (21, 22).

This change in subcellular localization of plasma membrane t-SNAREs suggests that membrane traffic pathways leading to the plasma membrane are fundamentally altered in epithelial cells during the course of the establishment of cellular polarity.

The formation of a polarized epithelial layer can be prevented experimentally by the inhibition of E-cadherin-mediated interactions between neighboring cells (1, 2, 13). Inhibition of calcium-dependent homotypic E-cadherin binding by withdrawal of high calcium concentrations in the medium keeps MDCK cells in a non-polarized state. It has been observed previously that, when grown in low-calcium medium, MDCK cells form large intracellular vacuoles that bear ultrastructural resemblance to the apical plasma membrane including the presence of microvilli and an associated actin cytoskeleton. This compartment was termed 'vacuolar apical compartment' or VAC (38). Similar vacuoles are found in a variety of carcinomas (16, 31, 40).

We studied the subcellular localization of plasma membrane SNAREs in MDCK cells grown under these conditions. We also used the mammary cells grown under conditions where they lose polarity, as described above. A high percentage (>50%) of the cells display one or more large vacuolar compartments that are positive for the endogenous apical marker protein gp135 and are indistinguishable in appearance from previously published VACs (. Plasma membrane t-SNAREs that normally localize to the apical domain (syntaxins 2, 3, SNAP-23), co-localize with gp135 in these VACs. In contrast, the normally exclusively basolateral syntaxin 4 is excluded from gp135-positive VACs. Instead, in addition to small vesicles, syntaxin 4 is found in larger structures that resemble VACs but exclude gp135. The SNAREs that are normally localized to both apical and basolateral plasma membrane domains (syntaxins 2, 11, SNAP-23), can be found in large gp135-negative structures (arrows) in addition to gp135-positive VACs.

Non-polarized MDCK and mammary cells stained with antibodies against syntaxin 4 and an endogenous 58 kDa basolateral plasma membrane protein (6.23.3). In addition to some plasma membrane-staining, both proteins are localized in large intracellular compartments that overlap significantly with each other (indicating that the syntaxin 4-positive compartment has a protein composition similar to the basolateral plasma membrane of polarized cells.

Together, the data suggests that although 'apical' and 'basolateral' t-SNAREs are localized intracellularly in non-polarized epithelial cells, they are nevertheless sorted to distinct compartments. These intracellular compartments resemble the respective plasma membrane domains due to the presence of 'apical' or 'basolateral' t-SNAREs as well as other plasma membrane marker proteins and an actin-based cytoskeleton.

The presence of normally apical and basolateral plasma membrane t-SNAREs in cognate intracellular compartments in non-polarized cells suggests that these SNAREs function in the membrane fusion of vesicles from incoming transport pathways that are equivalent to the plasma membrane-directed transport pathways in polarized cells. We investigated whether several proteins whose trafficking in polarized MDCK cells is well characterized are targeted to the syntaxin 3-positive VAC in non-polarized cells.

Together, these results strongly suggest that the intracellular VAC in non-polarized MDCK cells is a true cognate compartment to the apical plasma membrane in polarized cells as both contain an identical set of t-SNAREs and both receive membrane traffic from equivalent biosynthetic and endocytic pathways.

In polarized epithelial cells t-SNAREs distribute to intracellular compartments when cell polarity is lost or not yet established. To date the only functional information on the involvement of syntaxin homologues in plasma membrane traffic in polarized cells is available for syntaxin 3 which plays a role in transport from the TGN and from apical endosomes to the apical plasma membrane (17, 23). The exclusively basolateral localization of syntaxin 4 suggests that it is involved in polarized pathways to this domain. Therefore, this surprising result strongly suggests that membrane trafficking pathways that are normally directed to the plasma membrane in polarized epithelial cells undergo a fundamental shift towards intracellular compartments upon loss of cell polarity. This may have profound implications for our understanding of the pathogenesis of diseases involving a loss of epithelial polarity, e.g. the mistargeting of basement membrane proteins, proteases, integrins etc. that play a role in the pathogenesis of invasive and metastatic carcinomas (1), or the mistargeting of ion transporters, growth hormone receptors etc. in non-cancerous epithelial diseases such as polycystic kidney disease (25, 37). Also, during tubule formation - e.g. in kidney development - epithelial cells temporarily loose their cellular polarity while cell-rearrangements occur (29).

The presence of plasma membrane t-SNAREs on intracellular vacuoles in non-polarized cells indicates that plasma membrane proteins and secretory proteins are targeted into these vacuoles. Interestingly, we identified two distinct classes of vacuoles that can perhaps best be characterized by the presence of the apical t-SNARE syntaxin 3 or the basolateral t-SNARE syntaxin 4. Both compartments possess an actin cytoskeleton as shown by phalloidin staining. This distinguishes them from endosomes or other organelles which typically do not possess a prominent actin cytoskeleton but which is typical for the plasma membrane. Moreover, these compartments receive membrane traffic from cognate apical or basolateral pathways, respectively. This suggests that apical/basolateral sorting is preserved in non-polarized epithelial cells and leads to specific intracellular organelles. It has been found previously that non-polarized, fibroblastic cells also have the capability to sort apical and basolateral plasma membrane proteins (presumably in the TGN) and transport them on separate routes to the identical plasma membrane (26, 45). A

major difference between non-polarized cells of epithelial and non-epithelial origin may therefore be that in the former plasma membrane and secretory proteins are retained inside the cell rather than delivered to the surface.

The vacuolar apical compartment has been described and characterized previously in non-polarized MDCK cells and other epithelial cell lines as well as in a variety of carcinomas (3, 11, 38-40). In contrast, to our knowledge, the basolateral compartment that we identified here is a novel organelle that has not been described previously, perhaps because of the lack of availability of a marker protein such as syntaxin 4. What can be the possible function of "intracellular apical and basolateral plasma membranes"?

These compartments are observed in epithelial cells that have lost their cellular polarity temporarily (e.g. sparsely seeded cells that have not yet established cell contacts) or permanently (e.g. when cell contacts are inhibited or in tumor cells). It is likely that many plasma membrane or secreted proteins are still synthesized under these conditions. Re-directing these proteins (or lipids) to intracellular compartments will prevent their secretion or their display at the cell surface. This may be a protective mechanism as it would be undesirable or even harmful to the organism to have certain proteins secreted into the interstitial space by individual epithelial cells that have lost their cell contacts. A drastic hypothetical example may be accidental secretion of hydrolytic enzymes by non-polarized pancreatic acinar cells. Indeed, a variety of hydrolytic enzymes that are normally expressed at the apical plasma membrane of Caco-2 cells were found in VACs after microtubule disruption (11). The finding that neither the apical nor basolateral vacuoles appear to be degradative, lysosomal compartments indicates that proteins may be stored in them for later use once cell contacts have been re-established. This is supported by the observation that VACs can be rapidly exocytosed as a whole from MDCK cells after re-establishment of cell-cell contacts (39) or after raising the intracellular cAMP concentration (3). A basolateral equivalent of the VAC may be used to temporarily store proteins such as integrins. A major function of many epithelia is the directed transport of ions and solutes which is made possible by the proper apical or basolateral localization of transporters and channels. The intracellular storage of these channels and pumps may prevent accidental cell death due to excessive ion depletion or accumulation after loss of cell polarity. Interestingly, the CFTR chloride channel, while apical in polarized HT-29 cells, localizes to an intracellular compartment in non-polarized HT-29 cells (24).

In conclusion, we have shown that upon loss of cell polarity epithelial cells re-localize plasma membrane t-SNAREs and re-direct membrane trafficking pathways to intracellular cognate apical and basolateral compartments. This is likely to be a general phenomenon in epithelia and may play a fundamental role in the pathogenesis of epithelial diseases that involve a break-down of cell polarity.

7. Key Research Accomplishments.

* Localization of SNARE proteins changes as the cells change the polarization.
* Apical syntaxins localize to a VAC compartment in depolarized cells.
* Basolateral syntaxins localize to a novel intracellular compartment.

8. Reportable outcomes.

None so far.

9. Conclusions.

A basic property of mammary cells and other epithelial cells is that they are highly polarized. During oncogenesis polarity is lost, and in fact a hallmark of anaplasia is loss of polarity. Polarity requires that the machinery for transporting proteins to the correct apical or basolateral surface works correctly. We have begun to show how this is deranged in mammary carcinogenesis.

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11. Appendices.

None.